

United States Department of Agriculture  
Center for Veterinary Biologics  
Testing Protocol

SAM 207

Supplemental Assay Method for Potency Testing  
*Clostridium novyi* Type B Alpha Antigen

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## 1. Introduction

This Supplemental Assay Method (SAM) describes the method used to determine whether biological products containing *Clostridium novyi* type B alpha antigen can stimulate the production of satisfactory immunity as prescribed by the Code of Federal Regulations, Title 9 (9 CFR), Part 113.108. For products that require 2 vaccinations, rabbits are vaccinated twice 20-23 days apart and bled 14-17 days following the second vaccination. For products that require a single vaccination, rabbits are vaccinated and bled 34-40 days later. The serum is titrated by a toxin-antitoxin neutralization test, using mice as an indicator.

## 2. Materials

### 2.1 Equipment/instrumentation

- 2.1.1 Mixer, vortex-type
- 2.1.2 Centrifuge
- 2.1.3 Autoclave
- 2.1.4 Freezers, -20°C and -70°C
- 2.1.5 Refrigerator, 2°-7°C
- 2.1.6 Micropipettes, 100-µl and 1000-µl

### 2.2 Reagents/supplies

- 2.2.1 *C. novyi* type B alpha antitoxin, IRP 298
- 2.2.2 *C. novyi* type B alpha toxin IRP 425
- 2.2.3 Peptone diluent
- 2.2.4 Screw-top Erlenmeyer flask, 500-ml, with cap
- 2.2.5 Syringes, needle-locking, 1-cc, 10-cc, 20-cc, or 30-cc
- 2.2.6 Needles, 25- to 27-gauge x 7/8- to 1 1/4-inch, 20-gauge x 1-inch

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**2.2.7** Vacutainer® needles, 20-gauge x 1 1/2-inch and needle holder

**2.2.8** Serum separation tubes, 12.5-ml

**2.2.9** Pipettes, 2-ml, 5-ml, 10-ml, 25-ml

**2.2.10** Tips for micropipettes

**2.2.11** Ketamine hydrochloride, 100 mg/ml solution

**2.2.12** Xylazine, 20 mg/ml solution

**2.2.13** Water, distilled or deionized, or water of equivalent purity

**2.2.14** Polystyrene snap-top tubes, 17 x 100-mm

**2.2.15** Polystyrene screw-cap conical tubes, 17 x 120-mm

**2.2.16** Glass screw-cap tubes, 13 x 100-mm

**2.3 Test animals**

**2.3.1** New Zealand White rabbits, nonpregnant females, 4-8 lb (Eight rabbits are required per serial to be tested.)

**2.3.2** White Swiss nonpregnant female mice, 16-20 g (Five mice are required for each toxin-antitoxin mixture.)

**3. Preparation for the test**

**3.1 Personnel qualifications/training**

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware; and must have specific training and experience in the safe handling of clostridial toxins. Personnel must have specific training in the care and handling of laboratory rabbits and mice.

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### 3.2 Preparation of equipment and supplies

3.2.1 Sterilize all glassware before use.

3.2.2 Use only sterile supplies (pipettes, syringes, needles, etc.)

3.2.3 Operate all equipment according to the manufacturers' instructions.

### 3.3 Preparation of reagents

#### 3.3.1 Peptone diluent

Peptone (Difco)	8 g
NaCl, reagent grade	2 g
Water q.s. to	800 ml

Dissolve peptone and sodium chloride in water. Adjust pH to 7.2 with 1N sodium hydroxide. Fill a 500-ml Erlenmeyer flask no more than 3/4 full with diluent. Autoclave with caps loosened at 121°C for 25-30 minutes. Cool flasks and tighten caps. Store at 2°-7°C for up to 3 months.

#### 3.3.2 Preparation of *C. novyi* type B alpha antitoxin

1. *C. novyi* type B alpha antitoxin, IRP 298, contains 85 antitoxin units per ml (AU/ml) and has been standardized against the World Health Organization gas gangrene (*C. novyi*) International Antitoxin, equine origin. Each vial contains 4.5 ml of antitoxin.

2. Prepare a solution of *C. novyi* type B alpha antitoxin that contains 10 AU/ml by adding 1.0 ml of IRP 298 to 7.5 ml of peptone diluent in a 17 x 100-mm snap-top tube. Dispense in 2.25-ml amounts in glass 13 x 100-mm screw-cap tubes and store at -70°± 5°C until used.

#### 3.3.3 Preparation of *C. novyi* type B alpha toxin

Each vial of *C. novyi* type B standard toxin IRP 425 contains 2.3 ml of toxin. Store the toxin at -70°± 5°C until used. Any unused portion of IRP 425 may be

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refrozen one time in its original container or dispensed into individual 13 x 100-mm screw-cap tubes and frozen.

**4. Performance of the test**

**4.1 Vaccination of rabbits**

**4.1.1** Thoroughly shake each bottle of product and wipe the top with alcohol before filling the syringe.

**4.1.2** Vaccinate each rabbit subcutaneously in the shoulder region with not more than half of the largest recommended dose for any species indicated on the product label. Use 10-, 20- or 30-cc syringes fitted with 20-gauge x 1-inch needles to vaccinate the rabbits.

**4.1.3** For products that require 2 vaccinations, give the second vaccination 20-23 days after the first.

**4.2 Collection and preparation of rabbit serum**

**4.2.1** Collect blood from the test rabbits 34-40 days after vaccination (or 14-17 days after the second vaccination for products that require 2 vaccinations).

**4.2.2** Anesthetize rabbits for bleeding with a mixture of 1.32 mg/kg of xylazine and 8.8 mg/kg of ketamine hydrochloride. Give the anesthetic mixture by intramuscular injection.

**4.2.3** Use a 12.5-ml serum separation tube fitted with a 20-gauge x 1 1/2-inch Vacutainer® needle to collect blood from the heart. Collect approximately 12.5 ml of blood from each rabbit. Gently invert tubes 5 times. Let the tubes of blood sit at 22°-26°C (room temperature) for 30-60 minutes.

**4.2.4** Centrifuge blood at 1000 x g for 10-20 minutes at room temperature.

**4.3 Preparation of serum pools**

**4.3.1** Prepare a pooled sample using an equal volume of serum from at least 7 rabbits per vaccinated group

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(provided that, if more than 7 rabbits are bled per vaccinated group, then equal volumes from each rabbit are used for the serum pool). If less than 7 rabbits are bled, the test is invalid and must be repeated.

**4.3.2** The pooled sample may be held at 2°-7°C for up to 7 days if the test will be conducted within that time. If testing will not be completed within 7 days, store the pooled sample at -20°C or lower.

**4.3.3** Use 0.2 ml pooled serum diluted with 0.8 ml peptone diluent to test for 0.5 AU/ml of antitoxin.

**4.3.4** Use 0.1 ml pooled serum diluted with 0.9 ml peptone diluent to test for 1.0 AU/ml of antitoxin.

**4.4 Toxin neutralization**

**4.4.1 Preparation of standard alpha toxin**

1. Dilute the *C. novyi* type B alpha toxin to 1:10 by adding 1.0 ml of IRP 425 toxin to 9 ml of peptone diluent in a 17 x 100-mm snap-top tube. Further dilute the toxin to 1:62 by adding 1 ml of the 1:10 dilution to 5.2 ml of peptone diluent in a 17 x 100-mm snap-top tube. For the purpose of this test, the 1:62 dilution of IRP 425 is referred to as the standard alpha toxin.

2. A volume of 0.5 ml of standard alpha toxin and 0.5 ml of peptone diluent represents 0.1 L<sub>o</sub> dose. A volume of 0.8 ml of standard alpha toxin plus 0.2 ml of peptone diluent represents 0.1 L<sub>+</sub> dose.

3. For the purposes of this SAM, 0.1 L<sub>o</sub> dose is defined as the greatest amount of toxin that, when mixed with 0.1 AU, results in 100% survival of all mice inoculated intravenously (IV) with 0.2 ml of this mixture. The 0.1 L<sub>+</sub> dose is defined as the least amount of toxin that, when mixed with 0.1 AU, results in the death of 80%-100% of all mice inoculated IV with 0.2 ml of this mixture.

**4.4.2 Preparation of standard alpha antitoxin**

Thaw the 10 AU/ml *C. novyi* type B alpha antitoxin previously described (see **Section 3.3.2**). Further

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dilute the antitoxin to 1 AU/ml by adding 1 ml of the well mixed 10 AU/ml antitoxin to 9 ml of diluent in a 17 x 100-mm snap-top tube. Finally dilute the antitoxin to 0.1 AU/ml by adding 1 ml of the well mixed 1 AU/ml antitoxin to 9 ml of diluent in a 17 x 100-mm snap-top tube. For the purpose of this test, this 0.1 AU/ml dilution of antitoxin is referred to as the standard alpha antitoxin.

4.4.3 Product and standard alpha toxin

1. Mix a sufficient volume of standard alpha toxin and peptone diluent (0.5 ml of standard alpha toxin and 0.5 ml peptone diluent [0.1 L<sub>o</sub> dose]) for each serum pool and the L<sub>o</sub> control in a 17 x 120-mm screw-cap tube. Add 1 ml of each of the serum dilutions to 1 ml of this alpha toxin-peptone diluent L<sub>o</sub> mixture in 17 x 100-mm snap-top tubes. Mix each tube with a vortex-type mixer.
2. Let the mixtures sit at 22°-26°C (room temperature) for 1 hour.
3. Place tubes in ice.

4.4.4 Standard alpha toxin and antitoxin controls

1. Add 1.0 ml of standard alpha antitoxin (0.1 AU/ml) to 1 ml of the alpha toxin-peptone diluent (0.1 L<sub>o</sub> dose) mixture (see **Section 4.4.3**) in a 17 x 100-mm snap-top tube. Mix well with a vortex-type mixer.
2. Add 1.0 ml of standard alpha antitoxin (0.1 AU/ml) to a 17 x 100-mm snap-top tube containing 0.2 ml peptone diluent and 0.8 ml of standard alpha toxin (0.1 L<sub>+</sub> dose). Mix well with a vortex-type mixer.
3. Let the mixtures sit at 22°-26°C (room temperature) for 1 hour.
4. Place tubes in ice.



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#### 4.5 Inoculation of mice

4.5.1 Inject 0.2 ml of each standard test toxin-product antitoxin mixture into each of 5 mice.

4.5.2 Inject 0.2 ml of each standard test toxin-standard antitoxin mixture into each of 5 mice.

4.5.3 Inoculate all mice intravenously into 1 of the lateral tail veins. Use 1-cc syringes fitted with 25- or 27-gauge x 7/8- to 1 1/4-inch needles.

4.5.4 Always inoculate the mice receiving the standard test toxin-standard antitoxin mixtures (controls) **last**.

4.5.5 Mouse inoculations need to be completed within 1 hour of placing the toxin-antitoxin mixtures in ice.

4.5.6 The test is concluded 72 hours after the mice are inoculated.

#### 5. Interpretation of test results

##### 5.1 Criteria for a valid test

5.1.1 All 5 mice inoculated with the standard 0.1 L<sub>0</sub>/0.1 AU control mixture must survive.

5.1.2 At least 4 of the 5 mice inoculated with the standard 0.1 L<sub>+</sub>/0.1 AU control mixture must die.

**Note:** Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

##### 5.2 Interpretation of test results

5.2.1 If 5 of the 5 mice inoculated with the 1:5 diluted serum-toxin mixture (see **Section 4.3.3**) survive, the serum contains at least 0.5 AU/ml of *C. novyi* type B alpha antitoxin and the product is satisfactory.

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**5.2.2** If 5 of the 5 mice inoculated with the 1:10 diluted serum-toxin mixture (see **Section 4.3.4**) survive, the serum contains at least 1.0 AU/ml of *C. novyi* type B alpha antitoxin.

**5.2.3** The product is considered unsatisfactory if the serum pool from at least 7 rabbits contains less than 0.5 AU/ml of *C. novyi* type B alpha antitoxin.

## **6. Report of test results**

Report results of the test(s) as described by standard Section operating procedures.

## **7. References**

**7.1** Code of Federal Regulations, Title 9, Part 113.108, U.S. Government Printing Office, Washington, DC, 2005.

**7.2** History of reagents: *C. novyi* type B alpha antitoxin (IRP 298) was produced in goats at the National Veterinary Services Laboratories in Ames, Iowa, in April 1985.

**7.3** *C. novyi* type B alpha toxin (IRP 425) was produced at the National Veterinary Services Laboratories, Ames Iowa, in December 1994. The toxin was made from *C. novyi* type B alpha strain CN234.3. The culture was obtained from Wellcome Research Laboratories, Beckenham, England, on July 16, 1965. The number of passages is unknown.

## **8. Summary of revisions**

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **4.4.3** The wording was changed to add clarity.
- **4.4.4** The wording was changed to add clarity.
- Humane endpoint language was added.

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- Dilution/holding vessel sizes were added for clarification.
- The contact person was changed to Janet M. Wilson.

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